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14. ABSTRACT

We proposed to determine the changes in osteoblast differentiation *in vitro* when infected with multidrug resistant bacteria and the therapeutic effects of two well characterized antimicrobial peptides. For the first part of the study, an *in vitro* 3 dimensional (3D) model of primary human osteoblasts in a collagen scaffold was developed for infection. The 3D osteoblast cultures were infected with clinical isolates of multidrug resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Infection of osteoblasts revealed that all 3 species can invade osteoblasts and can be detected at 7 days post infection. Our preliminary findings suggest that these intracellular Gram negative bacteria can also reinfect healthy osteoblasts. Current studies are underway to investigate primary osteoblast differentiation and maturation when infected with these bacteria by gene and protein expression. The temporal gene expression of infected osteoblasts in the 3D model was studied by real time PCR. Initial work suggested changes in expression of proinflammatory cytokines and other genes that may ultimately delay osteoblast differentiation and maturation of infected cells. Due to intracellular survival of these pathogens, therapy with antimicrobial peptides was not addressed in the present work, as antimicrobial peptides that penetrate osteoblasts need to be developed. The outcome of our findings will contribute to accelerate treatment options and management of osteomyelitis due to drug resistant bacteria in the Wounded Warrior.

15. SUBJECT TERMS

Osteoblast, multi-drug resistant bacteria, infection

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INTRODUCTION:

We propose to determine the changes in osteoblasts *in vitro* when infected with multidrug resistant Gram negative bacteria. An *in vitro* 3 dimensional (3D) model of primary osteoblasts in a collagen scaffold will be utilized for the study. The 3D osteoblast cultures will be infected with clinical isolates of multidrug resistant *Acinetobacter baumannii* (AB), *Klebsiella pneumonia* (KP) and *Pseudomonas aeruginosa* (PA). Post infection, osteoblast differentiation and maturation will be determined in a temporal manner by gene and protein expression. Utilizing these models, the therapeutic effects of two well characterized antimicrobial peptides on osteoblasts infected with these three bacteria will be determined by bacterial clearance, osteoblast differentiation and maturation. The outcome of these findings will contribute to accelerate treatment options and management of osteomyelitis due to drug resistant Gram negative bacteria.

BODY:

Milestone 1: *In vitro* infection studies

Two different 3D models for osteoblast infections were proposed.
The first model was to establish a 3-Dimensional (3D) model for osteoblast infections utilizing primary osteoblasts in a collagen scaffold (BD biosciences) (n=4).

A second model was proposed which utilized hydroxyapatite discs with specific pore sizes purchased from DynCorp (UK). The company stopped its production of HA discs, and a suitable replacement was not found.

Therefore, the collagen scaffold model was selected for further studies. In this *in vitro* cell culture system, osteoblasts were to be cultured on a collagen scaffold (available from Becton Dickinson). The BD™ 3-dimensional collagen scaffolds are suitable for short- and long-term growth and differentiation of a variety of cell types, including osteoblasts (information from BD Biosciences). The 3D structure mimics the *in vivo* environment to induce tissue formation or to promote tissue repair.

For the proposed work, the collagen scaffolds were to be purchased by Beckton Dickinson, but the product was not available and was uncertain of its availability later. The problem was solved with a collagen scaffold model developed in the laboratory following modifications of Shen et al (1). The first year's work was dedicated to establish a working model with osteoblast cell line SaOs2. The first year's work described the establishment of the 3D collagen scaffold model with osteoblast cells line SaOS2 for infections. Work was completed sucessfuly to establish the model, and infections were performed with clinical isolates of multidrug resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* with SaOs2 cells prior to working with primary osteoblasts. The multiplicities of infection for all three strains were then optimized on the 3-D model with SaOs2 osteoblast cell line.

In the second year, work began with primary osteoblasts utilizing the established 3-D collagen scaffold model. To relate to war trauma, primary osteoblasts from healthy male subjects between the ages of 18-45 years were purchased from commercial sources (Lonza and Promocell Inc).

Milestone 2: Therapy with antimicrobial peptides

The second stage of the proposed study was to evaluate 2 synthetic antimicrobial peptides KSL and KSL-W on infected osteoblasts and study the therapeutic activities of these peptides.

Progress:

Milestone 1: *In vitro* infection studies

Primary human osteoblasts were cultured in appropriate media purchased from the commercial sources, with 10%FBS and 50ug/ml Ascorbic acid (Lonza and Promocell, Inc). Primary osteoblasts at the 3rd or 4th passage were used to load the collagen scaffolds (described in detail in the first report for collagen scaffolds with SaOs2 cells). Primary osteoblasts were evaluated for cell proliferation as described for SaOs2 cell line previously, and infection with bacteria, invasion assays were performed as for SaOs2 cell line.

Invasion of primary osteoblasts in the 3D model:

Scaffolds seeded with primary osteoblasts were used on days 12-13 for infection. The previous day before infection, media was replaced in wells with antibiotic free media. For infection, excess media removed on sterile Kimwipe from each scaffold, and moved to a 6 well plate. The appropriate number of bacteria was added onto the scaffold in a 10ul volume. The MOI for each bacterial species was established as 100:1 for *Acinetobacter baumannii* 5075, 20:1 for *Klebsiella pneumoniae* and 50:1 for *Pseudomonas aeruginosa*. Scaffolds were incubated for 2hrs at 37^{0}C. After 2 hours, each scaffold was rinsed in PBS and placed in a 24 well plate containing media with 10% FBS and 50ug/ml ascorbic acid and 100ug/ml polymyxin B. After 1 hour (t=0) for extracellular bacteria to be killed, and at each time point, scaffolds were rinsed in PBS, and digested in 1mg/ml collagenase for 30min, and a cell pellet was obtained after centrifugation at 2000RPM for 5 min in a micro centrifuge. Deionized distilled water was added to lyse the cell pellet. Lysates were plated on LB agar plates and the colony forming units (cfus) were enumerated the following day. Supernates from each well were plated out to ensure killing of extracellular bacteria (data not shown).

Preliminary studies on gene expression with very low MOI's of bacteria(data not shown) suggested that higher MOI's are needed to detect significant differential expression patterns. In order to increase the number of intracellular CFUs for *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in the osteoblasts, MOI's were adjusted to 20:1 and 50:1 bacteria:cells respectively. Intracellular CFU's were recovered at 7days post infection for the 3 bacterial species. The results of an infection of primary osteoblasts on the 3D collagen scaffold is shown in Fig.1.

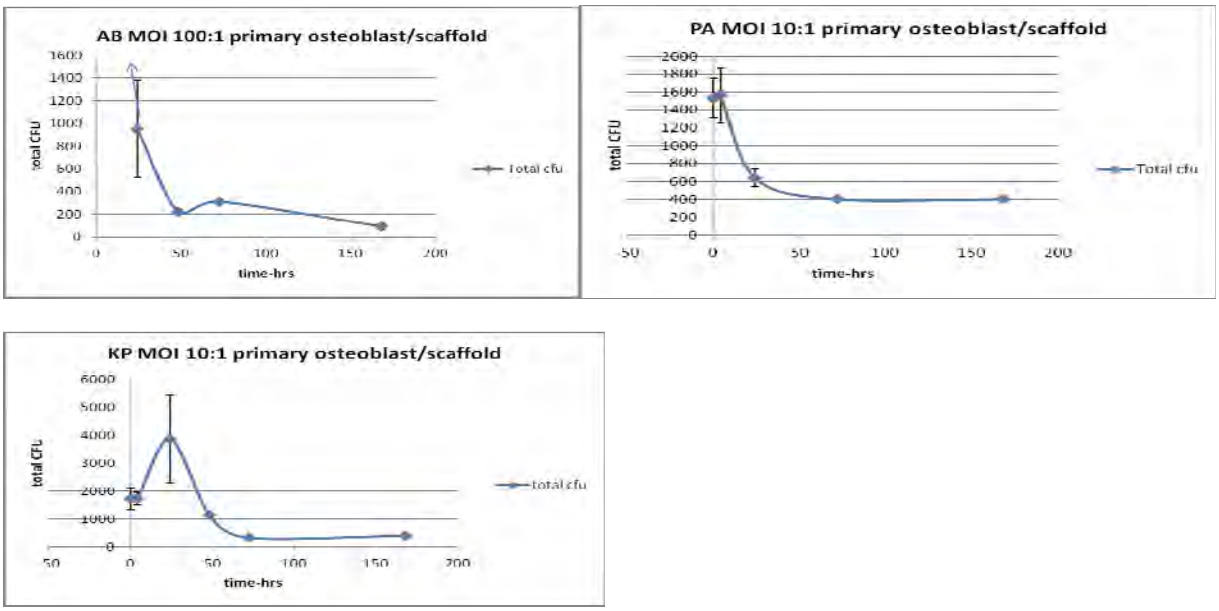


Fig 1. Recovery of intracellular bacteria (*Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP) and *Pseudomonas aeruginosa* (PA)) from primary osteoblasts at 7 days post infection from primary osteoblasts grown on a 3-D collagen scaffold (n=3).

The fate of these Gram negative intracellular pathogens after the death of the osteoblast is not known. The ability of released intracellular bacteria to reinfect new osteoblasts was investigated. In the invasion assays, to enumerate intracellular bacteria in osteoblasts, deionized water was used to release bacteria. This method, however, does not mimic the natural process of the death of osteoblasts. The fate of the intracellular bacteria after a more natural death of osteoblasts, thus creating a process which reflects the clinical situation, was utilized. These experiments were performed with monolayer SaOs2 cells. Work with primary osteoblasts is ongoing.

Methods:

Monolayer SaOs2 osteoblasts were infected with AB,KP and PA. Infection proceeded for 2 hours. After 2 hours wells were rinsed with PBS and media containing 100ug/ml of polymyxin B was added for 1 hour to kill extracellular bacteria. At 0, 24 and 72 hours post infection, infected cells were either lysed with distilled DI water, or whole unlysed cells were plated on agar plates after the addition of Trypsin-EDTA. After O/N incubation of plates at 37^{O}C the bacterial CFU's recovered from whole cells on agar plates were used to reinfect fresh SaOs2 cells. Results from a single experiment is shown.

The results suggest that lysis of osteoblasts is a better method to recover intracellular bacteria. The differences in cfus may be due to less survival of bacteria in dying host cells. Alternatively, intracellular bacteria released from dying host cells are not dispersed evenly so that the total cfu's detected is less.

Preliminary studies also suggest that all three species have the ability to reinfect healthy cells. More studies are ongoing to establish these findings.

Time course of infection (hrs)	**Number of intracellular (cfu/well)**					
	Lysed (control)	**Trypsinized (cells)**	**Lysed (control)**	**Trypsinized (cells)**	**Lysed (control)**	**Trypsinized (cells)**
	Acinetobacter baumannii		*Klebsiella pneumoniae*		*Pseudomonas aeruginosa*	
0	480	360	$3.6x10^3$	$1.8x10^3$	$21x10^3$	$13.5x10^3$
24	720	360	$4.5x10^3$	$3x10^3$	$3.9x10^3$	$6.9x10^2$
72	3x103	$2x10^3$	$8.2x10^3$	$3.9x10^3$	$2.7x10^3$	$8.4x10^2$

Table I. Recovery intracellular bacteria from osteoblasts after lysis of healthy cells with distilled water, or whole cells plated on agar for release of bacteria after death of cells.

	Intracellular cfu after reinfection at time zero
Acinetobacter baumannii	3.15x103
Klebsiella pneumoniae	18.9x103
Pseudomonas aeruginosa	38.4x103

Table 2. The ability of intracellular bacteria recovered from dead osteoblasts to re-invade healthy osteoblasts

Cytotoxicity assays for infection of osteoblasts with *Acinetobacter*, *Klebsiella* and *Pseudomonas* sp:

Method:

In order to look at the cytotoxicity in osteoblasts from infections, LDH assays were performed on the supernantes of infected osteblasts on the 3D scaffold. Infections were performed on primary osteoblasts on 3D scaffolds as described previously. At each time point, supernates from infected/uninfected samples were collected and stored at -80^0C. The supernates were then thawed and the LDH activity was determined according to the manufacturers instructions (Promega LDH assay). LDH levels were determined from a standard curve. The highest toxicity was detected in osteoblasts infected with *Klebsiella pneumoniae*. Higher levels of cellular toxocity was observed at each time point after infection with all 3 bacterial species compared to uninfected control.

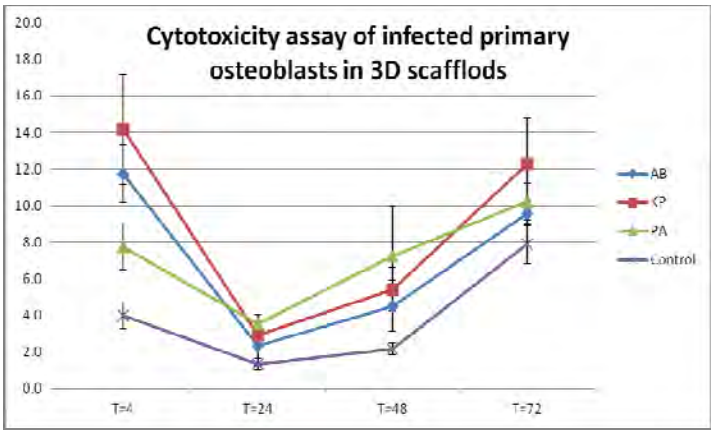


Fig.2. LDH measurements of supernatants of primary osteoblasts infected with bacteria over time. Temporal cytotoxicity of primary osteoblasts in 3D scaffolds when infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls (n=3). LDH released from the adhered cells in scaffolds was quantified. Y axis=Measurements of units/LDH/ml.

Studies on Osteoblast gene expression during infection with *Acinetobacter*, *Klebsiella* and *Pseudomonas* sp.

RNA extractions for gene expression study: Primary osteoblasts infections in the 3D model were then performed with the selected MOI's for each bacterial species for gene expression study. At present, two independent experiments have been completed, and the other 2 are ongiong.
Results of mean RT PCR gene expression for genes from 2 experiments are shown, and for this report statistical analysis was not performed.

Method:

Primary human osteoblasts at 3-4th passage on confluent flasks were harvested and scaffolds loaded as described previously. On day 12-13, infections with AB,KP and pA were performed as described previously. Infected primary osteoblasts on 3Dscaffolds were collected at 4,24,48 and 72 hours. A set of uninfected control osteblasts were included at each time point. 4-5 scaffolds at each time point were pooled together for extraction of total RNA.
Scaffold were digested with 1mg/ml collagenase, and a cell pellet was obtained after centrifugation at 2000RPM. 1ml of RNAzol was added to each pellet and resuspended. The samples were frozen at -80C until used for the gene expression study.
Total RNA was extracted with the Qiagen miniprep kit (Qiagen Inc). RNA was verified for concentration and purity by Nanodrop and Agilent 2100 Bioanalyzer.
From the literature, a panel of genes involved in osteblast differentiation and maturation were selected, and a custom real time PCR array was designed (Qiagen, SA Biosciences) to probe the infected/control samples. The list of genes for the custom array is shown in table 3.

GENE SYMBOL	GENE REFSEQ #
ALPL	NM_000478
VDR	NM_000376
BGLAP	NM_199173
CD11	NM_001797
FOXC2	NM_005251
RUNX2	NM_004348
ATF4	NM_001675
STAT1	NM_007315
OSTERIX	NM_152860
DLX3	NM_005220
DLX5	NM_005221
C-FOS	NM_005252
MSX2	NM_002449
TWIST1	NM_000474
HEY1	NM_012258
CDH4	NM_001794
CDH2	NM_001792
IBSP	NM_004967
LEF1	NM_016269
BMP2	NM_001200
BMP7	NM_001719
BETA ACTIN	NM_001101
GAPDH	NM_002046

Table 3. Gene list for RT^2 Profiler^{TM} custom PCR array for osteoblast infection

Total RNA from primary osteoblasts infected with AB, KP and PA were used to probe the arrays at t=4, 24, 48 and 72 hours post infection. An uninfected control was included at each time point. The PCR Arrays consist of sets of optimized real-time PCR primer assays with osteoblast differentiation maturation focused genes as well as appropriate RNA quality controls on 96-well plate format. The RT PCR array performs gene expression analysis with real-time PCR sensitivity and the multi-gene profiling capability of a microarray.

The RT PCR arrays were completed following the manufacturer's instructions (QIAGEN RT^2 Profiler^{TM}). Briefly, total RNA (500ng) was converted to cDNA according to the manufacturer's protocol (Qiagen Inc). cDNA was added to RT^2 qPCR master mix, and was added to the 96 well custom array which included the appropriate primers for each gene. PCR arrays were then

run in an ABI 7000 instrument. Data was uploaded according to instructions. The integrated web-based software package for the PCR Array System provided by Qiagen (SA BioSciences) automatically performs all $\Delta\Delta C_t$ based fold-change calculations from the uploaded raw threshold cycle data. The mRNA expression levels obtained for each gene were normalized to the mean expression of GAPDH and beta actin housekeeping genes by using the following equation: relative mRNA expression = $2^{-(\text{Ct of test gene} - \text{Ct of mean housekeeping genes})}$ (where Ct is the threshold cycle).

The results from two independent samples (n=2) are shown for this report. Two more independent samples (total n=4), and statistical analysis will be included at the completion of the project in December 2011.

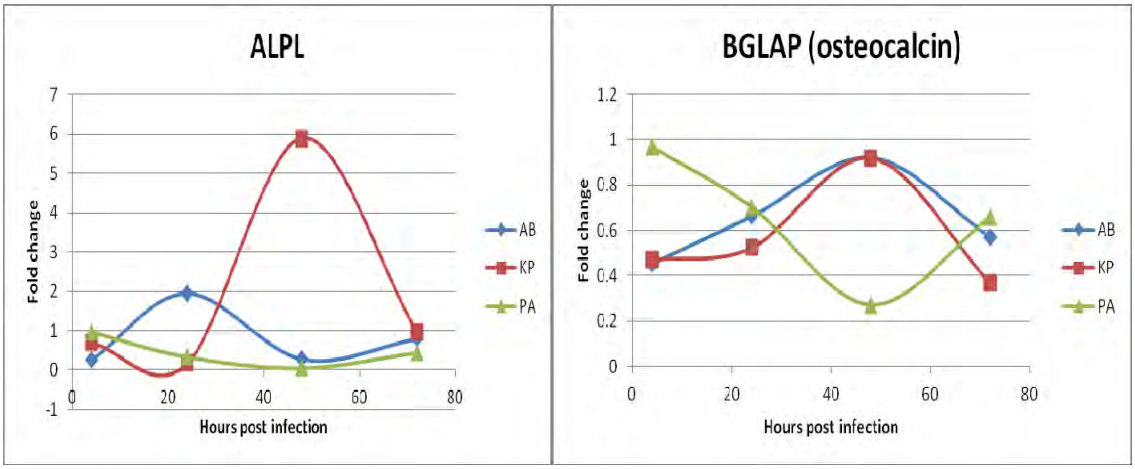


Fig 3. Temporal expression of osteoblast marker gene expression when primary human osteoblasts in a 3D scaffold are infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls (n=2).

Alkaline phosphatase (ALPL) is involved in osteoblast maturation, and a marker for bone metabolism and and *in vitro* studies have shown that expression of ALP increases with the differentiation of osteoblasts (2). When infected with PA, ALPL levels were consistently downregulated (Fig 3). ALPL levels varied when infected with AB and KP and by 72 hours post infection. Osteocalcin (BGLAP) constitutes the most abundant noncollagenous protein present in bone (3). Serum levels of osteocalcin correlate closely with bone formation (4) and, this marker has been widely used as an indicator of new bone formation in vivo (5). Osteocalcin production correlates with the onset of bone mineralization (6). Our preliminary data shows that the expression of osteocalcin is overall low during the course of infection, and downregulated by 72 hours suggesting a decrease in bone mineralization when infected with these bacteria. The lower expression levels of the bone markers during infection suggest that the intracellular presence of these bacteria in osteoblasts, (though in small numbers) may affect the normal differentiation and maturation of osteoblasts.

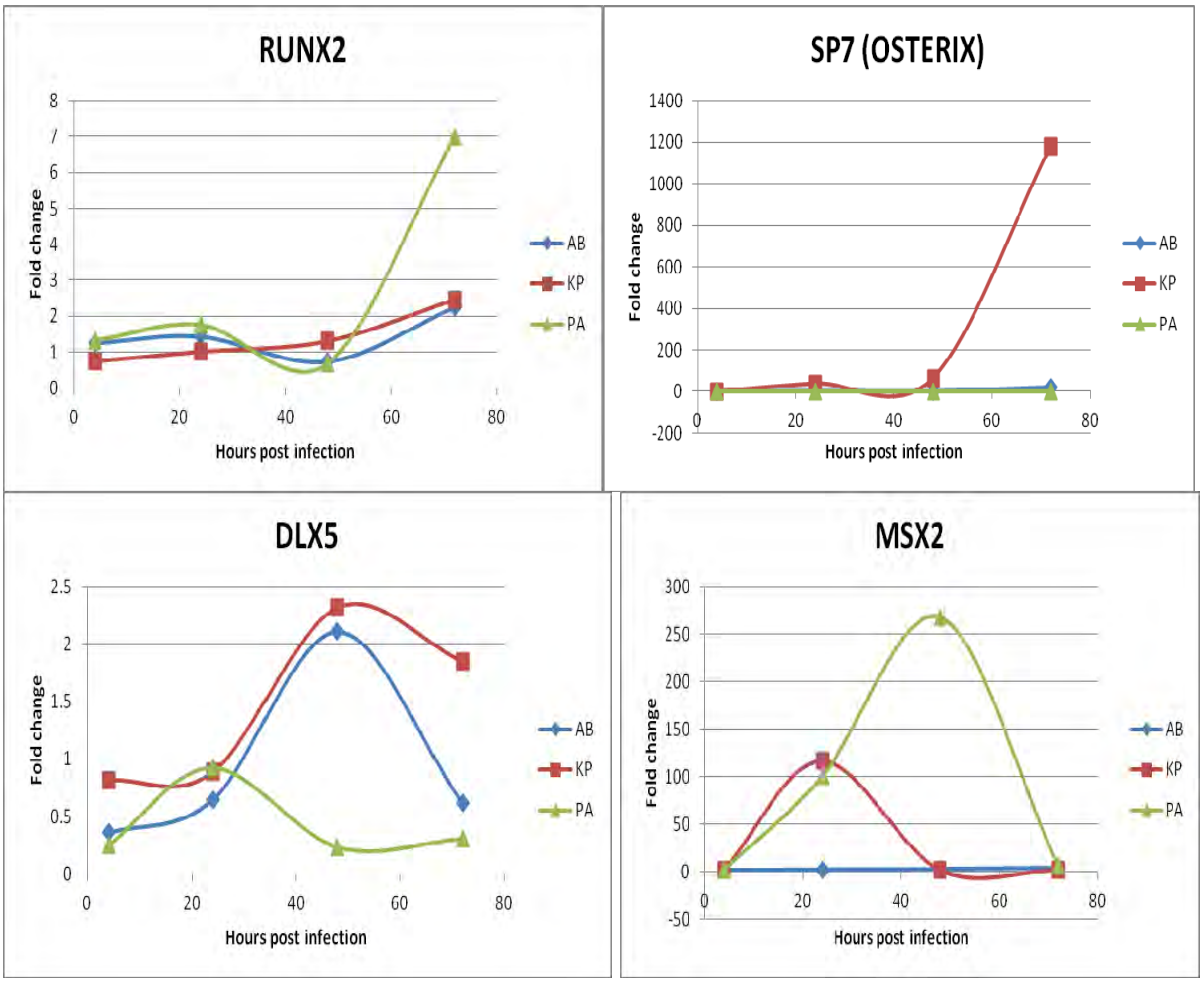


Fig 4. Temporal expression of osteoblast transcription factors in primary human osteoblasts in a 3D scaffold when infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls (n=2).

The transcription factors involved in osteoblast differentiation such as Dlx5, Msx2, Runx2, and Osterix were investigated for mRNA expression during infection.

Runx 2 is the vital osteogenic transcription factor, expressed in early skeletal development and persisting through subsequent stages of bone formation (7, 8). RUNX and Osterix (SP7) are two of the most essential genes for osteblast differentiation. During infection, the expression of RUNX2 was increased by 72 hours. Overall, infection with KP induced RUNX2 while for AB and PA, it was induced at 72 hours post infection (Fig 4). Osterix remained unchanged during infection to AB and PA and increased at 72hours post infection to KP (Fig4).

DLX5 is a bone inducing transcription factor, expressed in the later stages of osteoblastic differentiation, which is considered to be an upstream regulator of Runx2 and Osterix in the BMP-2 signaling pathway (9). Our preliminary data show that DLX5 expression was decreased

at 72 hours post infection to all three pathogens (Fig4). MSX2 plays a negative role in osteoblast differentiation, stimulating cell proliferation and suppressing osteogenic differentiation (8).
AB infection did not change the expression of MSX2 over time, and for both KP and PA, its expression increased, and then decreased by 72 hours post infection (Fig 4).

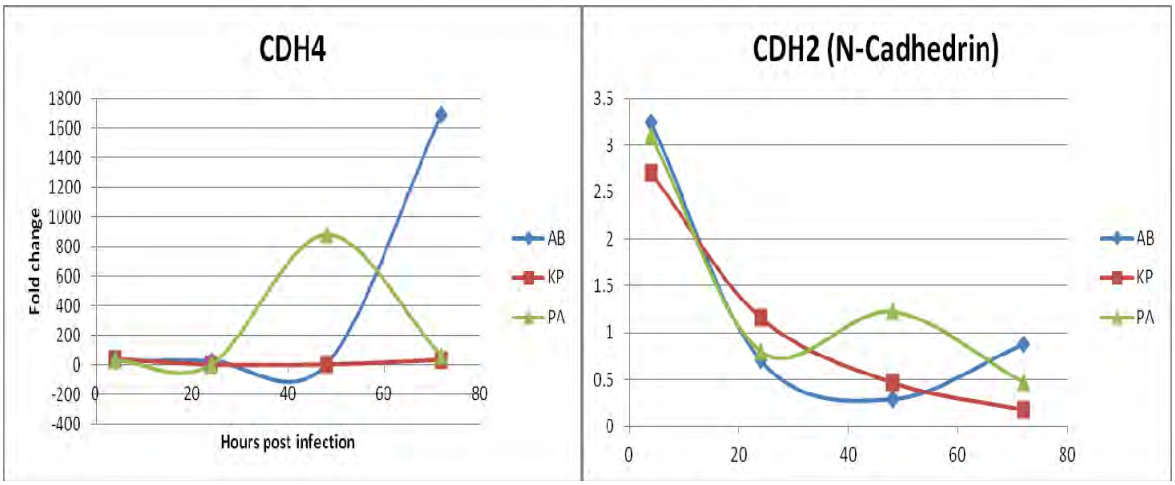
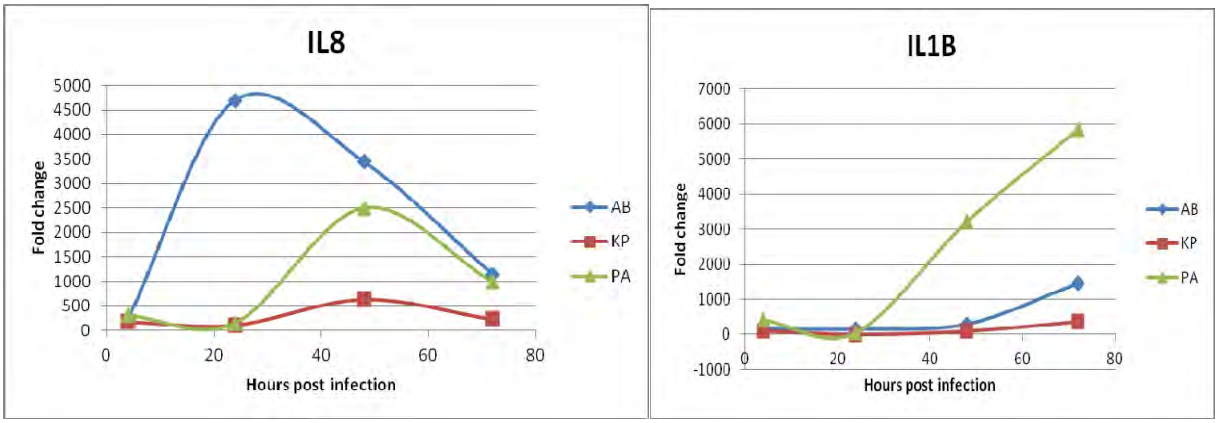


Figure 5. Temporal expression of osteoblast cell adhesion genes in primary human osteoblasts in a 3D scaffold when infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls (n=2).

Cell–cell adhesion by cadherins is essential for the function of bone forming cells during osteogenesis. Osteoblasts express a limited number of cadherins, including the classic N-cadherin, CDH2. The expression profile of N-cadherin in osteoblasts during bone formation *in vivo* and *in vitro* suggests a role of this molecule in osteogenesis. Preliminary data suggests that all three bacteria induced the expression of CDH2 (N-Cadhedrin) at 4hours post infection, but by 72 hours, it was down regulated (Figure 5).



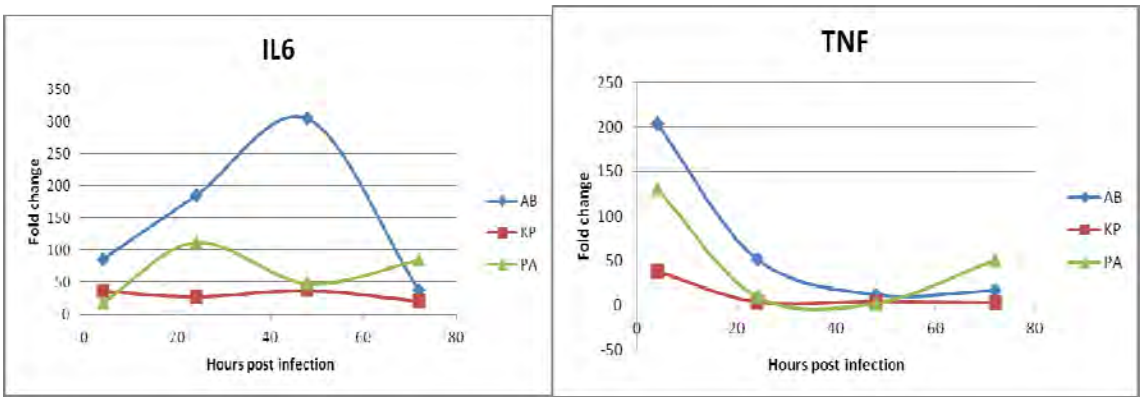


Figure 6. Temporal expression of cytokine/chemokine expression in primary human osteoblasts in a 3D scaffold when infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls (n=2).

Previous investigations on bacterial arthritis induced by *S. aureus* and *S. agalactiae* have suggested that TNFα, interleukin1β, and IL6, are involved in the pathogenesis of bacterial arthritis (10, 11). TNFa is known to be involved in osteoclastogenesis and stimulate osteoclast formation and differentiation (12). Both IL1 and TNFα appear to contribute directly to tissue damage through induction of the release of tissue-damaging enzymes by activation of osteoclasts (13, 14). Besides stimulating resorption, it is known that IL1 and TNF also inhibit bone formation *in vitro* (15-19). ILbeta was induced by PA after 24 hours and all three bacterial infections by 72 hours post infection (figure 6). TNFa expression decreased from 4 hours post infection and was increased for AB and PA infection at 72 hours. IL6 was elevated at all time points due to infection with AB, KP and PA. The neutrophil attractant chemokine IL8 expression increased and then decreased by 72 hours, but remained elevated for all 3 infections (Fig6). A recent study indicated that infection of Chlamydia pneumonia induced that maximum expression of proinflammatory IL8 (among other cytokines) at 72 hours post infection in SaOs2 osteoblast cell line (20). The expression of these chemokines/cytokines suggests that intracellular AB, KP and PA may elicit inflammatory responses in osteoblasts which in turn may hinder the differentiation and maturation of osteoblasts.
Currently, conclusions to be derived from this work is limited, and was not discussed in detail, due to the fact that the results presented here are that of temporal gene expression studies in 2 independent osteoblast samples. At the completion of this study, the final report will include statistical analysis and conclusions from a total of 4 independent samples.

Milestone 2: Therapy with antimicrobial peptides

The second part of the study was to evaluate antimicrobial peptide therapy to infection on osteoblasts in the 3D model. The current work revealed that all 3 bacterial species invade osteoblasts and can survive in osteoblasts as intracellular pathogens. As appropriate, therapies should then be focused towards the eradication of intracellular bacteria. The peptide KSL-W was evaluated for its ability to penetrate eukaryotic cells in invasion assays with AB, KP and PA and was found not successful in killing intracellular pathogens (data not shown).

Dr. Kai Leung of the U.S. Dental Research Institute at San Antonio, Texas is currently working on nanoparticle encapsulated KSL-W peptide (personal communication). An antimicrobial peptides formulation in nanoparticle encapsulation is more appropriate for treatment of intracellular AB, KP and PA infections of the osteoblasts.
Therefore, the current study did not address the therapeutic effects of KSL and KSL-W peptides on infected osteoblasts.

Challenges in the research project:

Work with primary osteoblasts is challenging as they grow very slow, and have limited ability to divide. To complete the work with primary osteoblasts an extension was requested and it is anticipated that the work will be completed by December 2011.
The proposed work was to test antimicrobial peptides to treat infected osteoblasts. The findings from this work indicated that all three species of bacteria can survive intracellularly in osteoblasts. The peptide KSL-W does not penetrate eukaryotic cell membranes, and future studies are needed to modify the peptide or encapsulate the peptide to reach the intracellular bacteria in order to treat infections due to these bacteria.

Key Research Accomplishments

- Establishment of a 3D collagen scaffold with primary osteoblasts to study osteoblast differentiation by gene expression.
- Developed a working 3D model of osteoblasts on a collagen scaffold to study the interaction of osteoblasts and bacteria.
- Significant finding- All three Gram negative bacteria that are associated with osteomyelitis in war wounds, namely, *Pseudomonas aeruginosa, Acinetobacter baumannii*, and *Klebsiella pneumoniae* invade osteoblasts and can survive intracellularly.
- Findings from this study suggest that intracellular *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* may be released from dying osteoblasts and have the ability to reinfect new osteoblasts.
- Preliminary findings suggest that several genes associated with osteoblast differentiation and maturation is impacted when infected with these Gram negative MDR bacteria and may negatively affect osteoblast maturation.

Reportable Outcomes:

- Oral presentation "*In Vitro* Osteoblast Model for Bone Wound Infections and Antimicrobial Therapy" at the Military Infectious Disease Research Program Defense Health Program enhanced (DHPe) Wound Symposium2-4 May 2011, San Antonio, TX
- Manuscripts in preparation

Conclusions:

This study resulted in an important finding in that Gram negative bacteria associated with war related osteomyelitis can survive intracellularly in osteoblasts.

In addition, preliminary investigations suggest that intracellular *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* have the ability to reinvade healthy osteoblasts. At the completion of this study, how infection with these three pathogens would impact the expression of several genes associated with osteoblast differentiation and maturation will be revealed.

"So what" section:
Osteoblasts and other cell types in vivo have a typically 3D structure from which they are able to migrate, attach and proliferate. In order to study osteoblasts in vitro, a natural matrix such as collagen to act as support for seeded osteoblasts is vital. In order to study the interaction of bacteria with osteoblasts, a 3D model of infection is essential as it has been reported that osteoblasts in a 3D environment is more physiologically relevant. An osteoblast 3D model for infections has not been reported in the literature yet.

The important finding from the present work is that *Acinetobacter, Pseudomonas and Klebsiella* invade osteoblasts. This work also suggests that infected dying osteoblasts may release viable bacteria that can reinfect other healthy osteoblasts suggesting a mechanism for reactivation of infection. The intracellular bacteria may also affect osteoblast maturation and differentiation, and therefore may delay bone wound healing. Most antibiotics used to treat osteomyelitis do not penetrate osteoblasts. These findings could potentially change treatment options for osteomyelitis in soldiers infected with these Gram negative pathogens.

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APPENDIXES:

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